LIVING MICROÖRGANISMS IN ANCIENT ROCKS

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In September, 1928, I reported in a note in *Science* that I had discovered living microörganisms in pre-Cambrian and other old rocks. In April, 1930, I reported, also in *Science*, an item on artificial bacilli in connection with which I stated that the work on microörganisms in rocks was being continued but that anthracite coal was then receiving exclusive attention, studies on other rocks being held in abeyance for the present. In this statement I shall confine myself entirely to my researches on anthracite coal which have thus far yielded the most striking evidence obtained in any of my studies on the existence of living microörganisms in ancient rocks.

I have been asked frequently since the inception of the studies under consideration to state what led me to make such an investigation when *a priori* one would not expect anything but negative results. My answer to this question is that for twenty years prior to the initiation of these experiments I had been accumulating more and more evidence on the persistence of the life of bacteria and bacterial spores for periods of forty years, as a maximum in the latter case, as authentic facts. The unabated virulence of pathogenic organisms grown from very old spores and the remarkable viability of bacteria from very old and very dry soils preserved in unopened bottles for about forty years have furnished me with much food for thought for well nigh a quarter of a century. Circumstances conspired to prevent me from subjecting my hypotheses to experimental test until nearly four years ago. Beginning with the oldest rocks on the face of the earth, the pre-Cambrian, and finding some evidence of bacteria in a living condition in them, I turned to rocks of other periods, and finally to anthracite coal because its origin promised for it the existence of a larger if not more varied flora. I argued

that if a spore or some other phase of a microörganism could persist alive for forty years there is no real reason why it might not do so for much longer periods even to millions of years.

EXPERIMENTAL TECHNIQUE

Since the technique of such an investigation is the most vital factor in determining the validity of its results I shall refer in this and in another part of my paper, in a detailed manner, to the experimental procedures employed. At the beginning of the work on anthracite coal I used commercial coal samples derived both from Wales and from Pennsylvania. Later I used specially collected samples of anthracite obtained at a depth of 1800 feet in a mine near Pottsville, Pennsylvania. The general procedure was as follows: A piece of coal about 2 to 3 inches in diameter was thoroughly scrubbed with soap and hot water and rinsed in distilled water (hot and cold). It was then dried with a paper towel and submerged in a beaker of Superoxol (30 per cent solution of H_2O_2). After five or six hours of exposure in Superoxol, the coal was transferred by means of a sterilized and very hot pair of tongs to a beaker of 95 per cent alcohol. After a few seconds of submergence in the alcohol it was grasped by another pair of sterile and very hot tongs and enveloped in the flame of a large burner until the alcohol had burned off, and then thrust into a large cast-iron, thoroughly sterilized mortar. The mortar was completely covered, pestle and all, except at its base, before sterilization, by two or three layers of clean absorbent cotton tied tightly to the mortar. The whole was sterilized in a hot air oven for no less than two days (at times for five and six days) at a temperature of 160° to 170°C. The time of exposure of the coal to the air as it went almost red hot from the flame into the mortar was perhaps a second. A flap in the cotton was raised for the purpose of transferring the coal to the mortar and immediately tied down again. By grasping the cotton covered and protected pestle and pounding, it was possible to crush the coal to powder in a minute or two. The mortar was then moved to another part of the laboratory (in more recent experiments to a specially constructed inoculation chamber). By raising the flap of cotton again and inserting a

large, long-handled, sterile and almost red-hot spoon into the mortar, it was possible to obtain a spoonful of coal powder for a quick transfer to an Erlenmeyer flask of sterile medium or to sterile water if a dilution experiment was made. No glassware was used before it had been sterilized for several hours at about 160°C. No medium was used without sterilization at least twice in the autoclave at 20 pounds pressure for periods of one and a half to two hours. In addition, media received an extrasterilization for half an hour to an hour just before being used. The cotton stoppers (after the medium had received the coal powder) were covered immediately with a piece of filter paper soaked in a 1:1000 $HgCl_2$ solution and the paper was fastened under the lip of the flask. After different intervals of incubation, usually after three or four days, the cultures were streaked or plated on an agar medium and were examined directly under the microscope. As was stated a moment ago, the foregoing procedure was generally employed in preparing the cultures; but many modifications of some features of the technique were employed. Some of these modifications will be mentioned presently. It is to be noted that about 100 experiments were performed which furnish the basis for this report. Obviously it is not possible here to do anything more than give a general summary of these numerous experiments but the crucial features of them will all be discussed.

A number of different types of media were employed in these investigations. The normal basis for all of them was coal extract made by heating powdered coal with tap water in the autoclave for ten to twelve hours at 20 pounds pressure and filtering and resterilizing. In some experiments, just the coal extract thus prepared was used as a medium. In other cases it was made up with 1 per cent peptone (the medium most widely used) or with starch. Other media used were 5 per cent glucose coal extract, sea water, and Bristol's algal medium.

GENERAL ACCOUNT OF RESULTS

Not all the replicate cultures in most series yielded growth but in some series they did. The rapidity of the appearance of growth varied with the medium and with the manner in which the coal

had been treated, as well as with the source of the sample. In general, however, the peptone media yielded growth in twenty-four to seventy-two hours (in 1 case, in five hours). In coal extract alone, growth might not be detectable until after two or three weeks of incubation. The types of organisms obtained showed only a slight range of variation. Most of them were short bacilli or cocco-bacilli and egg-shaped coccus forms, varying considerably in size and shape even in pure cultures. The tendency among all of them to occur in pairs was very marked. The colonies they produced on agar media at the first streaking were usually small and bead-like. Some of them were yellow to yellowish brown, others white, but in thin layers of growth, a distinctly blue color was always visible. In addition there were the ubiquitous blue, shadowy, somewhat larger colonies deep in the agar. The yellow colonies developed frequently as large surface colonies but quite commonly also as lenticular or cockade-shape colonies of smaller size, from which larger colonies would often develop. In some of the organisms there is a marked difference in appearance between a culture a day or two old and one of the same pure type several weeks old as exemplified strikingly in some of the microphotographs accompanying this paper. Moreover, the variability within a young pure culture was frequently most marked as regards both shape and size of the organism. Thus far I have not been able to demonstrate the existence in anthracite coal of molds, yeasts, or algae, but the number of experiments require manifolding to obtain indications of finality with regard to this last question. Apparently all of these organisms produce visible or invisible spores, or resting stages which serve the same purposes as spores. My results give fresh support, in ways which I have not time here to describe, to the conviction which I have often voiced during the last twenty years or more that all bacteria produce spores or bodies serving the same purpose, and that the distinction between spore and non-spore bearing organisms, while perhaps useful in certain ways, is not based on any critical study of resistant or resting stages in all forms. Certainly I regard the assumption that coccus forms do not produce spores, or something serving the same purpose as

spores, as being gratuitous and out of harmony with many interesting observations.

In a continuation of my discussion of the results obtained with my cultures, I come to another phase of my experimentation which involves a somewhat different technique from that which I have described, and which is fascinating in its far-reaching possibilities as regards this problem. Before adopting Superoxol as a bactericide for the sterilization of the exterior of coal or other rock samples intended for crushing, I had spent months in studying the effectiveness of all kinds of bactericidal agents and found nearly all of them useless for my purposes for one reason or another. The most effective I found was Superoxol and I shall state below how I checked the effectiveness of Superoxol before using it extensively. Nevertheless, it occurred to me that if heating a rock sample could accomplish the sterilization of its surface without killing all the bacteria inside, such procedure might serve as a good check on the Superoxol treatment and at the same time, might yield other important results regarding the heat resistance of the organisms under study. I carried out, therefore, many experiments with coal heated for various periods, both in the autoclave at 20 pounds steam pressure (about 115°C.) and in the hot air oven at a temperature of 160° to 170°C., principally by the latter method. Periods of heating ranged from two and one-half to twenty hours in the autoclave and from five to fifty hours in the hot air oven. Again, my results are too numerous to be given in detail in this brief communication but they may be summarized as follows: The surface of anthracite coal may be sterilized in ten hours or more in the hot air oven and in the autoclave in five hours without killing all the organisms inside of the coal even though heat penetration studies with thermocouples on the coal have demonstrated that the interior of such samples as I used in the hot air oven attains the temperature of the oven in about two hours after the sample is placed in the oven. It is still uncertain how effective the heating in the autoclave may be in point of lethal time for the interior of the coal. In the case of coal heated in the hot air oven, however, it has proved impossible to kill all the bacteria in the interior of the coal

sample even in a period of fifty hours under the conditions described above. Longer periods have not yet been tried. Moreover, it seems as if the longer periods of heating cause the organisms to grow more effectively in the cultures prepared from the crushed coal subsequent to the heating than do the shorter periods of heating. There is something very remarkable about this phenomenon which leads to possible implications in my mind which I prefer not to discuss for the present.

CHECKING THE TECHNIQUE EMPLOYED

In appraising and interpreting the results obtained by inoculating sterile media with crushed coal whose surface has been sterilized either by chemicals or by high heat a number of questions naturally arise with reference to the adequacy of precautions against contamination. I shall take up each of these questions separately in order to show how they are eliminated from consideration as determining factors in the results obtained. First, contamination of cultures from atmospheric sources during the placement of a sample of coal into the sterile mortar and during the distribution of the powder made by crushing such a sample into sterile media.

1. A sample of coal was broken into pieces not longer than ½ inch and placed in a sterile wide-mouth Erlenmeyer flask, stoppered with a cotton stopper, and put into the oven which was run at a temperature of 160° to 170°C. for a little over six days. After the flask was cooled at the end of that period, the coal was quickly transferred to a sterile mortar, as in the case of the routine technique with other samples. It was crushed to powder and distributed into six flasks of sterile 1 per cent peptone coal extract medium. After three or four weeks, an examination of these flasks directly and by transfers to agar slants of the same medium, showed no growth whatever. This experiment was repeated and the cultures thus prepared were plated, used for inoculation of slants and directly examined microscopically. Except for one or two fungi which appeared as air contaminants in these six containers, no microörganisms were found.

2. To check still further the experiments just described, a

sample of quartz sand was heated in the oven at a temperature of 160° to 170°C. for four days, using the same technique as was used with the coal. It was then transferred to a mortar, ground

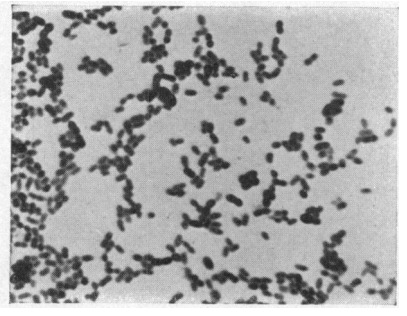

FIG. 1

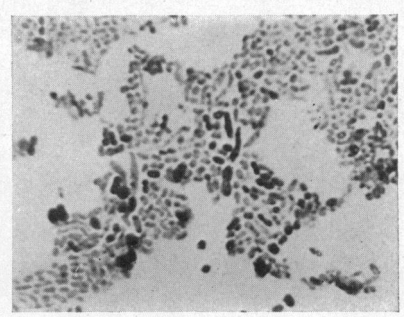

FIG. 2

FIG. 1. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM PENNSYLVANIA COAL, HEATED IN OVEN AT 160° TO 170°C. FOR FIFTEEN HOURS AND CRUSHED

Figures are in all cases magnified 1129 diameters

FIG. 2. SAME CULTURE THREE MONTHS OLD

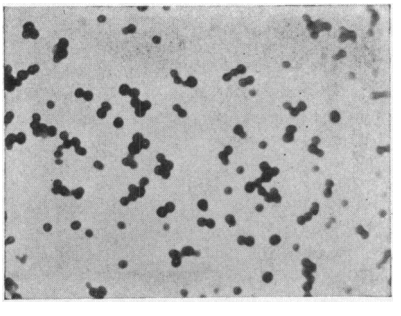

FIG. 3

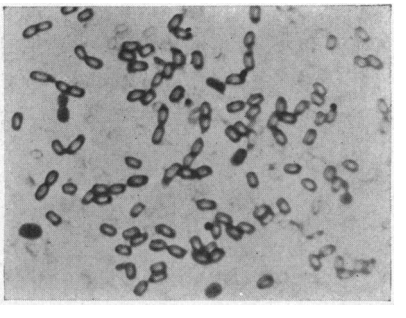

FIG. 4

FIG. 3. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM PENNSYLVANIA COAL, HEATED TEN HOURS IN OVEN AT 160° TO 170°C. AND CRUSHED, AFTER STAYING IN THE UNCRUSHED CONDITION FOR THIRTY-NINE DAYS IN COAL EXTRACT PEPTONE MEDIUM, WITHOUT SHOWING GROWTH

FIG. 4. SAME CULTURE THREE MONTHS OLD

with a pestle to some extent and inoculated into six flasks of sterile medium. Even after two months, these cultures showed no growth whatever and the medium remained perfectly clear.

Direct examination and streaking on agar gave no evidence of organisms.

3. Five samples of commercial coal derived from both Wales and Pennsylvania, of about the same size as those used in the

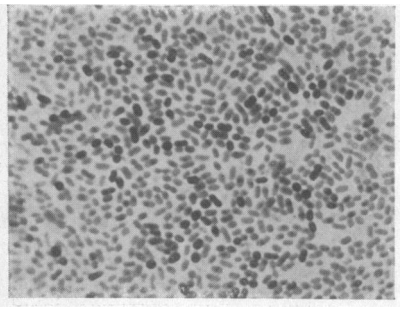

FIG. 5

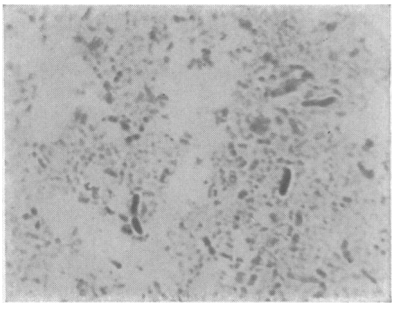

FIG. 6

FIG. 5. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM WALES COAL HEATED FOR FIFTEEN HOURS IN THE OVEN AT 160° TO 170°C. AND CRUSHED

FIG. 6. SAME CULTURE THREE MONTHS OLD

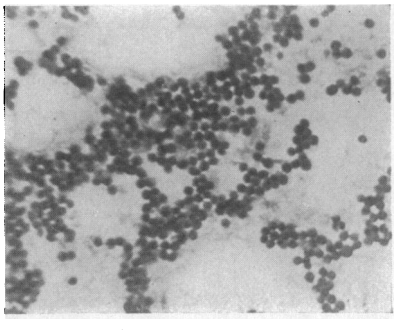

FIG. 7

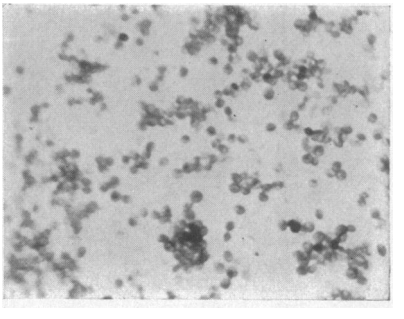

FIG. 8

FIG. 7. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM WALES COAL HEATED IN THE OVEN FOR FIVE HOURS AT 160° TO 170°C. AND CRUSHED, AFTER HAVING BEEN ALLOWED TO REMAIN IN AN UNCRUSHED CONDITION FOR 55 DAYS IN COAL EXTRACT PEPTONE MEDIUM, WITHOUT SHOWING GROWTH

FIG. 8. SAME CULTURE THREE MONTHS OLD

routine experiments described, were placed in Superoxol for periods varying from three to six hours, then transferred to alcohol. The sample was taken out of the alcohol, the alcohol was

burnt off, and the sample kept enveloped in the large flame for two or three seconds longer and quickly transferred to sterile peptone coal extract medium in a wide mouth Erlenmeyer flask. To protect the cotton stopper from dust, it was covered by a piece of filter paper soaked in a solution of $HgCl_2$, concentration 1:1000. After periods varying from one month to three months, it was found that the media into which these coal samples were placed remained clear and transfers from them showed no microorganisms. The different samples were then crushed as in the regular procedure which I have described, and distributed into

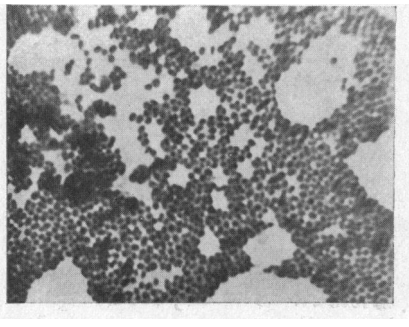

FIG. 9

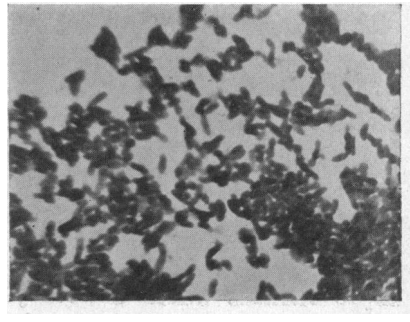

FIG. 10

FIG. 9. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM WALES COAL AFTER COAL WAS EXPOSED IN SUPEROXOL FOR FIVE HOURS AND FIFTEEN MINUTES AND CRUSHED

FIG. 10. SAME CULTURE THREE MONTHS OLD

fresh sterile culture media. In the case of every piece of coal thus treated, the crushed material yielded growth in more than half of the cultures made from it. In some cases, growth was obtained in all of the cultures made from a given piece of coal.

4. Five samples of commercial coal derived from Wales and Pennsylvania were heated in wide mouth Erlenmeyer flasks in the hot air oven at temperatures varying between 160° to 170°C. for periods of five to ten hours. At the end of the period of heating used in each case, the flasks were cooled and sterile 1 per cent peptone coal extract medium was poured quickly over the coal samples, sufficient to submerge them. The cotton stopper

was then replaced, covered with a protecting filter paper soaked in $HgCl_2$ as before, and placed in the incubator as were the other samples. After periods varying from one month to three months in the incubator, the samples were removed and it was found that the medium remained perfectly clear in all cases and an examination of the supernatant liquid, directly, and by means of slants, showed no microörganisms present. The samples were then removed from the flasks, dipped into alcohol, the surface again burnt in the alcohol flame and additionally in the open flame of a large burner, and then transferred to a sterile mortar and crushed as in the case of the other series. Sterile culture media inoculated with coal powder thus prepared gave growth in more than half of the several cultures made from each piece of coal. In one or two cases, all of the cultures thus prepared showed growth.

The organisms found in the cultures described as prepared from pieces of coal whose surface only had been sterilized, either by chemicals or by heat, were limited to two or three forms closely similar, if not identical with the cocco-bacillus and very short diplo-bacillus which I have just described as being found quite uniformly in anthracite coal. This applies to all coal samples examined, whether from commercial coal, or from the special samples obtained from a great depth in a coal mine in Pottsville, Pennsylvania.

5. As a further check on my technique, it seemed desirable that if possible, plate cultures with or without dilution be made immediately after a sample of coal was crushed, so that it might be possible to observe the production of colonies, if any, from the dormant cell forms in the coal. This seemed particularly desirable since it has never been possible for me to observe directly in powdered coal any vegetative or even spore forms of microörganisms. Twelve samples of coal, mostly derived from the heart of the special sample from Pennsylvania, were sterilized on the surface, in half of the cases with Superoxol and in the other half by heating ten hours in the oven at 160° to 170°C., crushed, transferred into thoroughly sterilized and filtered tap water, shaken for three to five minutes, and quantities varying from 1 to 2 cc. were distributed immediately into sterile Petri dishes and sterile

peptone agar poured over the suspensions in the plates. In nearly all of these series which were so arranged as to be used in quantitative determinations of the numbers of microörganisms in the coal, it was found after the proper periods of incubation that no colonies developed; but after the coal suspension in tap water from which these dilutions were made had been incubated for a few days to three weeks, plates similarly made yielded colonies in nearly all cases. It should be remarked that these colonies were produced from organisms which had developed in nothing but a suspension of coal powder in sterile tap water. They had apparently multiplied enormously in that medium and yet the cells from which these numerous organisms were derived were incapable in agar of producing colonies in nearly all of the plates made in the original quantitative experiment. Apparently the immediate transfer of a suspension from the coal powder in tap water to agar inhibits the development of the dormant cells into vegetative forms. This striking phenomenon was checked further in some of the series of experiments in this category by transferring from 1 to 5 cc. of the coal-powder-tap-water suspension immediately after it was made, to liquid sterile media in Erlenmeyer flasks. After a few days, in which the media at times remained clear in these new cultures, and at times became slightly turbid, plates poured from these transfer cultures always yielded colonies which in turn showed organisms of the same types as those described earlier in this paper. This proved true when the most favorable medium was employed, namely, 1 per cent peptone coal extract, for the transfers from the original suspension, or when pure distilled-water-clear-coal-extract itself was used as a medium, or when 1 per cent peptone-starch-coal-extract was used as a medium. Incidentally, the several series of plates which showed no colony growth serve as another check on my technique as regards the plating of any of the culture series.

DISCUSSION AND CONCLUSIONS

The literature available on studies of coal carried out in various ways does not, so far as my search has gone and this has admittedly not been complete, reveal any case of an investigator who

has indicated the possibility that microörganisms found in coal may be a few living relics of originally abundant flora in the material from which the coal was derived perhaps as long as one or two hundred million years ago. Most of the investigators who have concerned themselves with studies on the bacterial flora of coal do not even suggest that the organisms which they found in coal are anything more than modern bacteria which have come into the coal from the outside in very recent time. Such seems to be the case in the investigations of Schroeder, of Galle, and of Potter. On the other hand, Lieske, in a very brief note discussing his investigations, dismisses with a word the suggestion that the microörganisms which he found in coal and which he regarded as belonging to the species *Bacterium liquefaciens-fluorescens* and perhaps one additional form, could possibly be representatives of any ancient bacteria which have lived over in the coal from the time of its formation. In other words, therefore, I am making a claim which so far as I know, has never been made in connection with the studies of bacterial flora of rocks, (practically all of these have been limited to coal, and there have not been many) namely, that the microörganisms found in coal are actually survivors, imprisoned in the coal at the time it was formed, from material which originally was probably very rich in microörganisms since it was peat-like in nature. It is my view that here and there scattered through the masses of the coal measures an occasional spore or some similarly resistant resting stage of a microörganism has survived the vicissitudes of time and circumstance and retained its living character, its power to develop into a vegetative form, and its power to multiply when conditions are rendered propitious for it. I do not regard it as at all necessary that a living cell like a spore destroy itself by respiring away its own substance in the course of time. I believe that it is quite possible for a cell like a spore to remain in a state of suspended animation with respiration not occurring at all, and I believe that in a thoroughly desiccated condition of its protoplasm as would be the case with a spore locked up in coal, that a state of suspended animation is quite a conceivable and even probable phenomenon in nature. If one assumes such a hypothesis and

further, the hypothesis that spores have survived only in small numbers and occur sporadically within the coal mass or in the mass of other rocks, then one can readily see how results could be obtained similar to those which I have obtained in some one hundred experiments which I have carried out with anthracite coal.

From the studies which I have made of the literature which bears on the observation of microörganisms in coal, I feel that no investigator besides myself has really taken the precautions necessary to a proper study of microörganisms of coal, if the question of their having lived over from the time of the formation of the coal is held in view. I have employed such rigorous methods for preventing contamination that I have felt at times that there was a possibility that some organisms which were present in the coal samples used were actually destroyed by the drastic methods which I used for the sterilization of coal surfaces and of apparatus.

Before I close, I desire to discuss one other factor which bears on the validity of my conclusion. Anyone who has thought about a problem of this sort must necessarily have surmised that organisms found in anthracite coal today, and perhaps also in other rocks, might have gained entrance into such rocks very recently through crevices or pores which the rocks might contain. In fact, Lieske has expressed the view that the organisms which he found in anthracite coal were washed down into the coal measures from the surface of the ground which covers the coal, by seepage water and other surface water percolating downward. In the course of my experiments, therefore, I addressed myself to studies or to special precautions which might resolve that difficulty. As regards precautions, I may say that after working for a long time with commercial coal, I had collected for my use specially large blocks of coal as above stated from a deep mine near Pottsville, Pennsylvania. The gentleman who was kind enough to collect these samples for me, Mr. Raymond C. Johnson, Research Chemist of the Philadelphia and Reading Coal and Iron Company, assures me that the samples were collected from a place in the mine at a depth of 1800 feet from the surface, where there is no

evidence whatever of percolating water in or near the point at which the samples were obtained, and yet cultures made from this coal give results practically identical with those made from commercial samples of coal. Such evidence, however, may not be as satisfying as direct studies upon the penetrability of coal samples by chemical substances and by living cells. I therefore made a study of the permeability of coal to the following substances: water, sodium chloride, in solution, mercuric chloride in solution, potassium dichromate in solution, and eosin. I used these substances because it was possible to employ a test for each of them after the coal had been submerged in the solution in question. The tests were made by submerging carefully washed chunks of coal in these substances and keeping them in that submerged state for one to three weeks. I found that all of these substances penetrate coal fairly readily and particularly in certain areas of the coal which seem to be more permeable than others. Owing to the limitation of time I shall not go into a discussion of the tests used, especially in view of the fact that they all showed positive penetration of these substances into the coal. It must be remarked, however, that these substances are composed of molecules which even in the case of the largest of them, namely the eosin molecule, are very small compared to bacterial cells, and for that reason I started an experiment to determine whether or not a sample of coal which had been thoroughly heated and sterilized could be penetrated by bacteria from a suspension of the organism in a proper medium. The description of this experiment is as follows:

Two pieces of coal were carefully washed with soap and water and thoroughly rinsed in distilled water, and each placed in a wide-mouth Erlenmeyer flask of 500 cc. capacity. The flasks were stoppered with cotton and placed in the oven at 160° to 170°C., and kept there for six days. At the end of that time the flasks were taken out of the oven and allowed to cool, and sterile 1 per cent peptone coal extract was poured into one of the flasks sufficient to submerge the coal sample. Into the other flask a suspension of a coccus isolated from one of the coal samples studied earlier in the same medium was poured over the coal

sample. Filter paper soaked in a solution of $HgCl_2$ of 1:1000 concentration was then adjusted over the cotton stopper and tied tightly under the lips of the flasks so that any dust or organisms would be prevented from touching the stopper. The flasks were then placed in the incubator at 28°C. and kept there for over three months. At the end of that time the coal sample which had been submerged in sterile peptone coal extract and which latter had remained perfectly clear and uncontaminated throughout that period, was removed to a sterile mortar, after dipping in alcohol and burning of the surface, and crushed. The coal powder thus produced was inoculated into several flasks of sterile peptone coal extract medium. Except for two or three organisms which were clearly air contaminants found in these cultures, they remained perfectly clear and streaking and plating from them yielded entirely negative results.

In the case of the sample of coal which had been submerged in a suspension of a pure culture of the coccus derived from coal, the following procedure was used: It was removed from the medium which was of course turbid, owing to the growth of the coccus, thoroughly rinsed in distilled water, dried, and placed in Superoxol for six hours. At the end of that time, it was removed from the Superoxol, placed in alcohol for a minute, removed from the alcohol again and enveloped in a large gas flame until the alcohol had burnt off, and perhaps for a second or two beyond that time. The sample was then quickly placed in a sterile mortar and crushed, and the powder was distributed into several flasks of sterile peptone coal extract medium. Only one of the several flasks produced any growth, and that after several days of incubation. The growth was a contaminant which bore no resemblance to the coccus in a culture of which that coal sample had been submerged for three months. The other flasks remained clear and after some time, were all plated on peptone coal extract agar. A few colonies all told, perhaps not more than eight or ten, were found on these several plates. It is obvious that if the coccus in which the coal sample was submerged had penetrated to any extent at all into the coal that each culture made from the crushed sample would have shown heavy growth and each plate

made from these cultures would have shown colonies too numerous to count.

The conclusion seems irresistible therefore, that particles as big as a coccus are too large to penetrate the coal, either through crevices or through microscopic pores as do the chemical substances which I discussed above.

The idea of Lieske, therefore, that organisms from surface water could have penetrated into anthracite coal seems to me to be untenable. In view of this, I return to the conclusion which I presented above, namely that the microörganisms which I found in the anthracite coal are descendants directly from cells which have lain dormant there from the time of the coal's formation, which according to one method of the geologist's reckoning, would be fifteen million years, and according to another method, from one to two hundred million years.